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PCR Based Idendification and Discrimination of *Caranx rhonchus* (Pisces, Carangidae) Based on Nuclear and mtDNA Sequences

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Abstract: PCR based nuclear and mitochondrial DNA markers are used to clarify cases of misidentification of adult individuals of fish species with morphological similarities. This problem prevails in the case of false scad (*Caranx rhonchus*) overlap geographically and has similar morphology with Turkish species of the genus *Trachurus*. Hence, the nuclear DNA/mitochondrial DNA approach was used for *C. rhonchus*, to identify and discriminate, it from *Trachurus* sp. coexist. Polymerase Chain Reaction (PCR) amplification of the nuclear 5S rDNA gene is a suitable method for differentiating between *C. rhonchus* and *Trachurus* sp. On the other hand, a PCR-RFLP technique, which involves digesting of the whole mitochondrial cytochrome b gene with XcmI and BsrGI restriction enzymes to generate DNA profiles, was used to identify of *C. rhonchus* and discriminate it from three *Trachurus* sp. This PCR-RFLP analysis is simple, rapid and reliable and hence can be regularly used to discover fraudulent substitution among economically important *C. rhonchus* and *Trachurus* sp. Results have confirmed the usefulness of these techniques to distinguish and genetically identification of morphologically similar carangid species.

Key words: Genetic identification, *C. rhonchus*, false scad, 5S rRNA, Cyt b, PCR-RFLP

INTRODUCTION

Authenticity of fresh, frozen and cured fish products has become a very important issue related with fraudulent substitutions of species in commercial products. Due to an increasing range of fish species and a variety of fish products appeaering in the market, it is inevitable to develop and innovate current methods used for fish species identification. Labelling of respective fish species is necessary from an aspect of prevention of adulteration because fish species of different value and price may be fraudulently substituted (Hubalkova *et al*., 2007). Two genera of carangid fish are the most significant: *Caranx* and *Trachurus*. False scad (*Caranx rhonchus*) are often marketed as horse mackerel (*Trachurus* sp.) owing to the greater popularity of and higher consumer demand latter species. On the other hand, according to the annual catch statistics derived from Turkish Statistical Institute, fishing mortalities of *Trachurus* sp. were totally 13.115 tons for the Northeastern Mediterranean and Aegean seas, but statistical data for *C. rhonchus* are unavailable. This may be attributed to False scad (*Caranx rhonchus*), having a considerable population size, are quite often confused morphologically with *Trachurus* sp. or are consciously often marketed as horse mackerel (*Trachurus* sp.) owing to the greater popularity of and higher consumer demand for these species.

Numerous analytical techniques have been developed for fish species identification and discrimination. Species identification based on protein analysis had been formerly widely used and regarded as a reliable method (Rehbein *et al*., 1995; Hsieh *et al*., 1997). Moreover, standart protein-based identification methods, such as electrophoresis, are less reliable for speciation of processed fish products than for fresh or frozen products because DNA is less affected by food processing technologies than proteins are (Ram *et al*., 1996; Quinterio *et al*., 1998). Therefore, DNA-based techniques offer an alternative approach to for species identification and have recently started to be applied towards a wide variety of fish, including closely related species belonging to the same family and genus (Davidson, 1998; Bossier, 1999; Lockley and Bardsley, 2000). Direct DNA sequencing is the most reliable method for identification and/or discrimination; however, sequencing can become very expensive and it cannot

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easily be applied to samples suspected or known to contain >1 species. Numerous genetic approaches to the determination of species identity are alternative techniques that utilize DNA polymorphism of a region of mitochondrial DNA by Polymerase Chain Reaction (PCR), such as direct sequence analysis of the amplified fragment, or Restriction Fragment Length Polymorphism (RFLP) analysis (Carrera *et al*., 1999), has strengthened owing to a combination of reability and relatively rapid and inexpensive sample screening. Many of these genetic approaches have been based on highly conserved regions of the mt genome such as the cytochrome b gene and the 5S and 16S ribosomal RNA genes (Meyer, 1994). Mitochondrial DNA (mtDNA) has proven to be useful in molecular phylogenetic studies because evolutionary relationships can be inferred among higher levels, between recently divergent groups, populations, species and even individuals (Avise, 1994). The mtDNA is of maternal inheritance and has no recombination in all vertebrates, so that the sequence of mtDNA is more conservative (Rokas *et al*., 2003). Mitochondrial DNA is a broadly used genetic tool and one of its advantages is the high copy numbers of the mitochondrial genome compared with nuclear genome within a cell. Therefore, mtDNA-based methods can be applied to small amounts of tissue, such as eggs or processed samples (Karaiskou *et al*., 2005).

The mitochondrial cytochrome b (cyt b) gene is indicates to be of ancient origin (Esposti *et al*., 1993) and shows a relatively high mutation rate and sufficient point mutations to enable discrimination of a wide variety of fish, even closely related species belonging to the same families and genera (Chow *et al*., 1993; Ram *et al*., 1996; Quinterio *et al*., 1998; Lindstrom 1999; Russel *et al*., 2000; Jerôme *et al*., 2003). Polymerase Chain Reaction (PCR) amplification and restriction enzyme analysis of the cytochrome b gene have also been used for identification of fish species.

Nuclear 5S ribosomal DNA (5S rDNA) is present in multiple copies tandemly repeated in the eukaryotyc genome, constituting a very large multigene family. The 5S rDNA gene is appropriate candidates which may allow direct species identification and genetic discrimination of related species by PCR amplification without the need of later sequencing or digestion with restriction enzymes (Pendas *et al*., 1994; Belkhiri *et al*., 1997; Asensio *et al*., 2001). This method is rapid, reliable and can be applied to the detection of fraudulent or unintentional mislabeling of these species in the market.

C. rhonchus (Geoffroy Saint-Hilaire, 1817) and 3 species of the genus *Trachurus coexist* in Aegean and Northeast Mediterranean Seas. The morphological similarity among *C. rhonchus* and *Trachurus* sp. (also especially *T. mediterraneus*) complicate especially identification of juvenile *Caranx rhonchus* and they separated from *Trachurus* sp. as morphological features cannot always be easily distinguished. Hence, *C. rhonchus* is in easily confuse with *Trachurus* sp. and thus, combined nuclear DNA (5S rDNA) and mtDNA (cytb) approach can be used for the identification of mislabeled *Caranx* products in order to detect and eliminate commercial fraud. These approaches could also provide a new method of egg identification as all 3 species have eggs similar to those of horse mackerel and they cannot be properly identified with the current methods. Furthermore, the development of genetic markers for fish species identification and separation will increasingly be required for legislation designed to regulate regional fisheries and products in Turkey.

The aim of this research was to develop simple DNA-based methods for identifying or discriminating *C. rhonchus* from Turkish *Trachurus* sp. based on restriction site polymorphism analysis of mitochodrial cyt b gene and a PCR-based agarose gel electrophoresis of the nuclear 5S ribosomal DNA gene. These approaches have been used successfully to discriminate false scad from horse mackerels.

MATERIALS AND METHODS

Seventy five specimens of *C. rhonchus* were collected 4 localities, which were represented, in the Aegean and North-eastern Mediterranean Seas: Izmir, Bodrum, Antalya and Iskenderun (Fig. 1). Samples were frozen at -20°C and transferred in dry ice to the laboratory. Later, the caudal fin was dissected from each individual and stored in -20°C until use.

DNA extraction, PCR amplification and electrophoresis: Total genomic DNA was extracted from samples using Promega Wizard® Genomik DNA Purifikasyon Kiti (Promega Corporation, Madison, USA) according to the manufacturer's instructions. The concentration of extracted DNA was determined using Agilent 8453E UV-visible Spectroscopy System (Agilent Technologies, Santa Clara, CA, USA) and extractions were stored at 20°C. The mitochondrial DNA cytochrome b gene was amplified using primers H15149 (5-AAACTGCAGCCCCT CAGAATGATATTTGTCCTCA-3) and L14841 (5'-AAAA AGCTTCCATCCAACATCTCAGCATGATGAAA-3') (Kocher *et al*., 1989). Amplifications were carried out in 50 µL reaction volumes containing 0.5-0.6 mg of genomic DNA, 5 mL of 10 µL reaction buffer, 50 mM dNTPs, 1 U of Taq DNA polymerase (Go Taq, Promega Madison,

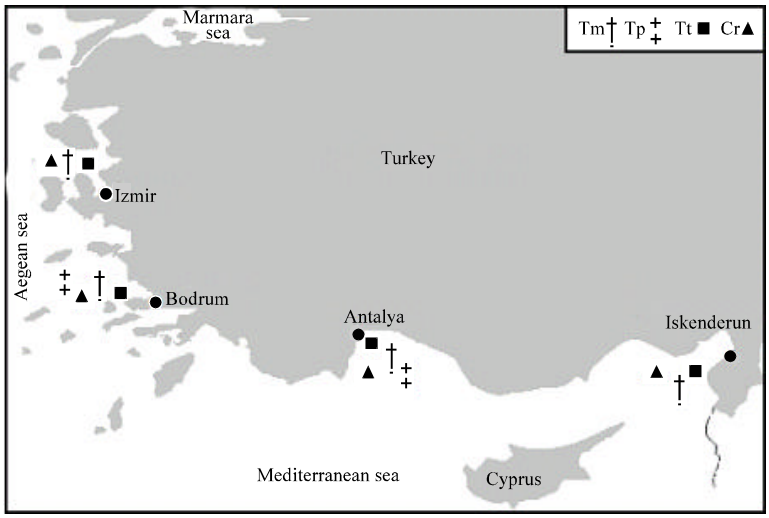

Fig. 1: Map of the sampling locations

WI, USA) and 50 mM $MgCl_2$, 20 pmol of each of the primers. DNA was amplified in a BioRad Thermal Cycler (MJ Research, Inc., Walthan, MA, USA). The following cycling parameters were used: 1 cycle of initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec, primer extension at 72°C for 1 min and a final 7 min elongation at 72°C. The size of the PCR products was checked against a 100-bp DNA ladder (Gibco BRL, Madison, WI, USA) in 1.5% agarose gel, run in 1X TAE buffer and stained with 0.5 mg mL^{-1} ethidium bromide.

Nuclear 5S rDNA: The set of primers used for PCR amplification of the 5S rDNA gene in *C. rhonchus* and *Trachurus* sp., were designated as follows: 5SF (5'-TACGCCCGATCTCGTCCGATC-3'.) forward and 5SR (5'-CAGGCTGGTATGGCCGTAAGC-3'.) revers primers, designed by Pendas *et al*. (1995). These primers have already been used to amplify a whole unit of the 5S rRNA gene from species of *Trachurus* (Karaiskou *et al*., 2003, 2005) and from different fish species (Pendas *et al*., 1995; Céspedes *et al*., 1998; Rodriguez *et al*., 2001). Double-stranded amplifications were carried out in 50 µL reaction volumes containing 0.5-0.6 mg of genomic DNA, 5 mL of 10 µL reaction buffer, 50 mM dNTPs, 1 U of Taq DNA polymerase (Go Taq, Promega Madison, WI, USA) and 50 mM $MgCl_2$, 20 pmol of each of the primers. DNA was amplified in a BioRad Thermal Cycler (MJ Research, Inc., Walthan, MA, USA). The following cycling parameters were used: 1 cycle of initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec, primer extension at 72°C for 1 min and a final 7 min elongation at 72°C. DNA was amplified in a BioRad Thermal Cycler (MJ Research, Inc., Walthan, MA, USA). Thirty two cycles were performed, with the following step-cycle profile: initial denaturation at 95°C for 3 min, strand denaturation at 94°C for 30 sec, primer annealing at 60°C for 45 sec and primer extension at 72°C for 30 sec and a final 7 min elongation time at 72°C. Electrophoresis of a 10 µL portion of the amplification product was performed for 1 h at 100 V in a 1.5% agarose gel, run in 1XTBE buffer and the gel was stained in a solution containing 0.5 mg mL^{-1} ethidium bromide. The size of the PCR products was checked against a 100-bp DNA ladder (Gibco BRL). DNA fragments were visualized by UV transillumination and analyzed using BioDoc Analysis System (Biometra, GmbH, Germany).

Direct sequencing of the cytochrome b gene: PCR amplifications were sequenced using BigDyeTM terminator cycling protocols. PCR products were purified using ethanol precipitation and run on an Automatic Sequencer (ABI 3730×1) by a contract laboratory. Sequencing of the 5' end of cyt b was carried out using the primer L14841 (Kocher *et al*., 1989). Sequences with ambiguous sites were resequenced from the 3' end. PCR product purification and DNA sequence analysis were performed by Macrogen Inc. (Seoul, Korea).

Endonuclease digestion and PCR-RFLP analysis of mitochondrial cytochrome b gene: The restriction endonuclease XcmI (CCANNNNN$^{?}$NNNNTGG) and BsrGI

(T^?GTACA) (New England Biolabs, Inc., USA) were tested for restriction analysis of the amplified PCR products. Digests were performed in a 10 µL volume with 100-200 ng of the amplified DNA, 5 U enzyme and 1:10 diluted manufacturer's 10X digestion buffer. Digestion mixtures were incubated for 2 h at 37°C. The resulting fragments were separated by electrophoresis in a 1.5% agarose gel containing 10 µg mL^{-1} ethidium bromide for 30 min at 80 V. The sizes of the resulting DNA fragments were estimated with a 100-bp DNA ladder (Gibco BRL, Madison, WI, USA).

RESULTS AND DISCUSSION

Identification and discrimination of *C. rhonchus* using the nuclear 5S rDNA marker: The 5S rDNA is a very good candidate for identification/discrimination of related species because of a high interspecific variability and interspecific size differences in 5S rDNA are sufficient to distinguish different fish species (Pendas *et al*., 1995; Céspedes *et al*., 1998; Karaiskou *et al*., 2003, 2005). The nuclear 5S rRNA gene, which was selected as an first candidate for identification of *C. rhonchus* was amplified and the size of amplified fragments was checked in agarose gel. As shown in Fig. 2, amplification of *C. rhonchus* gave a only one band of 410 bp, while the 5S rRNA patterns of the other three *Trachurus* sp., as suggested by Karaiskou *et al*. (2003, 2005), are totally different, *T. trachurus* giving a double band of 210 and 230 bp, *T. mediterraneus* a double band of 410 and 430 bp and *T. picturatus* a pattern of 2 bands at around 210 and 350 bp. The single PCR product of about 410 bp was observed in false scad.

For the 5S rRNA gene, the absence of intraspecific variability and the presence of interspecific variability had already been reported by Karaiskou *et al*. (2003) in *Trachurus* sp. Similarly, in the present research, various specimens of *Caranx rhonchus* were studied, representing the broad geographical range of the species. Overall, 58 samples were analysed, covering the distribution area of *C. rhonchus* in Turkish waters. Intraspecific polymorphism within false scad was not detected, because the electrophoresis gel pattern obtained was the same within or between populations of the same species. This technique is probably, the most simple PCR procedure which was developed for specific identification of false scad (*C. rhonchus*), based on the amplification of clearly different-sized products from the 5S rDNA gene. For these reasons, the nuclear 5S rDNA gene can serve as a reliable species-specific nuclear marker for *C. rhonchus*.

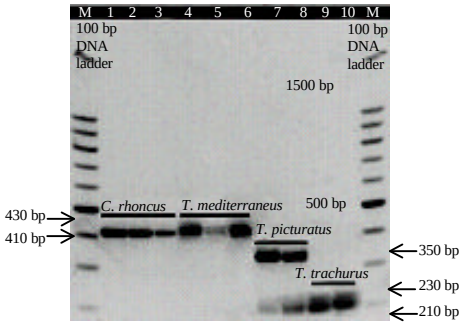

Fig. 2: Electrophoretic analysis of 5S rDNA gene PCR products obtained from false scad (1-3), mediterranean horse mackerel (4-6), blue jack mackerel (7, 8) and atlantic horse mackerel (9, 10), using oligonucleotides 5SF and 5SR. M indicates kb plus DNA ladder for molecular weight marker

Direct sequencing of the cytochrome b gene: The whole cytochrome b gene (1141 bp) was generated from 58 *C. rhonchus* (22 from 2 locations in the Aegean Sea and 26 from 2 locations in the northeastern Mediterranean Sea). Totally, four different haplotypes were determined for *C. rhonchus*, with haplotypes *C. rhonchus* 1 and *C. rhonchus* 3 (Fig. 3) shared by all studied populations and the rest (*C. rhonchus* 2 and *C. rhonchus* 4) being unique and found only in the Izmir and Iskenderun samples, respectively. These sequences were compared with sequences of cyt b gene previously obtained for the 3 *Trachurus* sp. by Bektas and Belduz (2008). One hundred and thirty nine sites were found capable of discriminating between *C. rhonchus* and the 3 *Trachurus* sp.

Differentiation and identification of *C. rhonchus* by RFLP analysis of cyt b: To determine the appropriate restriction enzymes that is discriminated and identified PCR products of *C. rhonchus* from the 3 *Trachurus* sp., the sequences of the mitochondrial cytochrome b gene from related species were aligned and analysed to identify restriction sites within the gene that were reliability different between the species. It was determined that 2 restriction endonucleases (Xcm I and BsrGI) were the most useful for comparing related species because both enzymes have a specific recoqnition sequence and restriction site in false scad and absent in horse mackerel. This interspecies sequence comparison of related species revealed that one XcmI and BsrGI restriction site are present only in *C. rhonchus*, but not horse mackerel (Fig. 3). The restriction enzyme XcmI produced 2 fragments of 136 and 1005 bp from the PCR products of

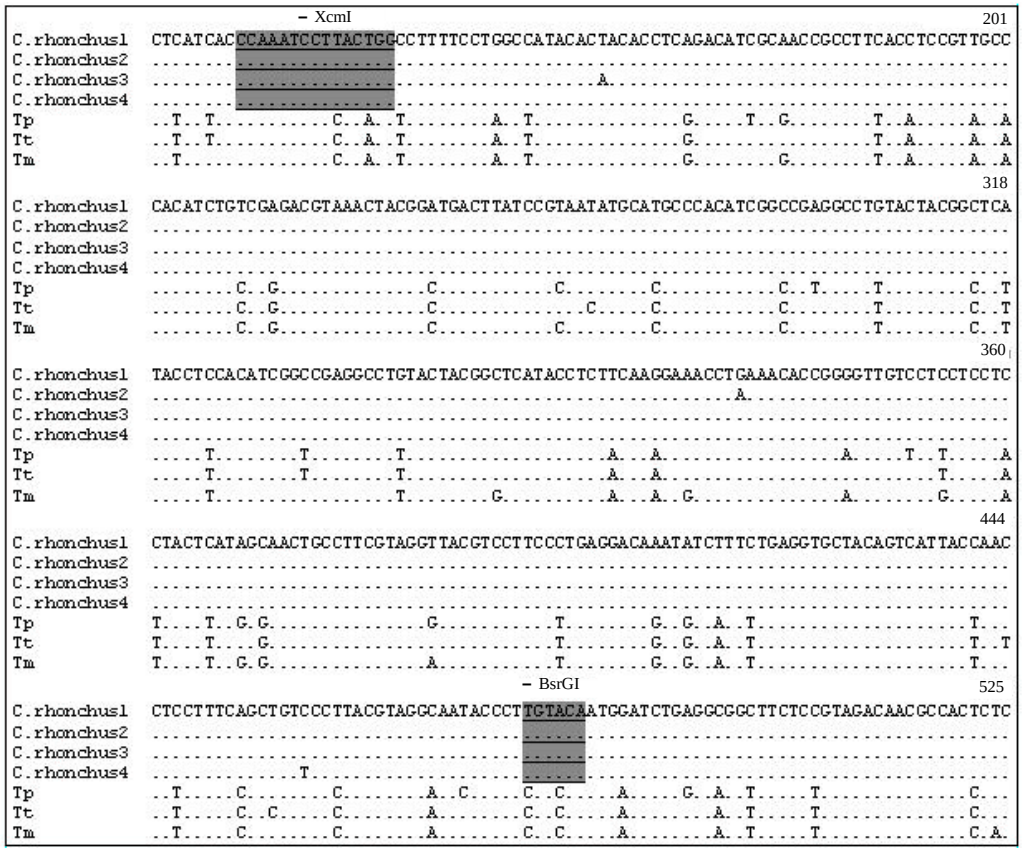

Fig. 3: Aligned DNA sequences of cyt b gene for *C. rhonchus* (4 different haplotypes) and the 3 *Trachurus* sp. (*T. mediterraneus*, *T. trachurus* and *T. picturatus*), after Bektas and Belduz (2008). Restriction recognition sites for specifc restriction enzymes with diagnostic capability are shaded

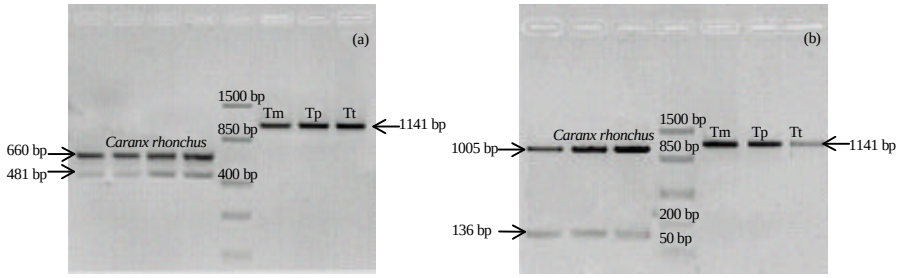

Fig. 4: PCR-RFLP analysis of the mitochondrial cytochrome b gene after digestion with BsrGI (a) and XcmI (b): *C. rhonchus* (Cr), *Trachurus mediterraneus* (Tm), *Trachurus picturatus* (Tp) and *Trachurus trachurus* (Tt)

C. rhonchus, whereas its restriction site was not present in *Trachurus* sp. (Fig. 4). Additionally, for BsrGI digestion, *C. rhonchus* gave a pattern of 2 fragments of around 481 and 660 bp, while the PCR product in the 3 *Trachurus* sp. was not cleaved, because no recognition site was present (Fig. 4). Additionaly, False scad and *Trachurus* sp. did not show intraspecific polymorphism for the 2 restriction endonucleases tested. Therefore, the 2 restriction endonucleases were useful for the discrimination of *C. rhonchus* from *Trachurus* sp. As intraspecific variability can impair the efficiency of the methodology, a large number of fish used in order to confirm the absence of intraspecific polymorphism of the cytochrome b gene sequence. The results were confirmed

Table 1: The origin and number of *C. rhonchus* species analysed at mt cyt b and nuclear 5S rRNA genes

Locality	5S rRNA	Cyt b
Izmir	12	12
Bodrum	10	10
Antalya	16	12
Iskenderun	20	13
Total	58	47

by analysing 47 *C. rhonchus* for XcmI and BsrGI, throughout the geographical distribution of the species (Table 1). The patterns obtained by agarose gel electrophoresis with the restriction enzymes showed band sizes that concordanted the observed sizes for the restriction fragments inferred from the sequence analysis. Some techniques can be performed to identify unknown samples to species. However, these methods are a costly and time-consuming. An alternative approach to sequencing is the analysis of PCR fragments with endonucleases to detect interspecific restriction fragment length polymorphism (Meyer *et al*., 1995; Ram *et al*., 1996). The PCR-RFLP of the cyt b gene has been used previously to differentiate between *Gadoid* fish species (Aranishi *et al*., 2005; Hubalkova *et al*., 2007) and to identify billfish species (Hsieh *et al*., 2005), flatfish species (Sanjuan and Comesaña, 2002), marine fish fillets (Cocolin *et al*., 2000).

The utility of nuclear and mitochondrial DNA markers for identification and discrimination of samples belonging to closely related species were investigated in the present study. These methods are extremely useful as it has the potential to detect a wide range of fish species following the development of suitable PCR-RFLP profile can be applied as a rapid means of comparing samples. To improve the reliability of these approaches, it would be useful to expand the number of fish species for which PCR-RFLP profiles are avaliable. As a result, this simple PCR amplification provides a powerful method to detect conscious as well as fraudulent substitution of these species, particularly in the market of false scad.

CONCLUSION

PCR based methods could be used to clarify cases of misidentification of adult individuals of fish species with morphological similarities and overlap geographically. The PCR-RFLP analysis is simple, rapid and reliable and hence, can be regularly used to discover fraudulent substitution among economically important *C. rhonchus* and *Trachurus* sp. The present results have confirmed the usefulness of these techniques to distinguish and genetically identification of morphologically similar carangid species.

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